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Dynamic changes in cytoskeleton proteins of olfactory ensheathing cells induced by radiofrequency electromagnetic fields FREE

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ABSTRACT

Several evidences have suggested the ability of radiofrequency electromagnetic fields to influence biological systems, even if the action mechanisms are not well understood. There are few data on the effect of radiofrequency electromagnetic fields on self-renewal of neural progenitor cells. A particular glial type that shows characteristics of stem cells is olfactory ensheathing cells (OECs). Herein, we assessed the non-thermal effects induced on OECs through radiofrequency electromagnetic fields changing the envelope of the electromagnetic wave. Primary OEC cultures were exposed to continuous or amplitude-modulated 900 MHz electromagnetic fields, in the far-field condition and at different exposure times (10, 15, 20 min). The expression of OEC markers (S-100 and nestin), cytoskeletal proteins (GFAP and vimentin), apoptotic pathway activation by caspase-3 cleavage and cell viability were evaluated. Our results highlight that 20 min of exposure to continuous or amplitude-modulated 900 MHz electromagnetic fields induced a different and significant decrease in cell viability. In addition, according to the electromagnetic field waveform, diverse dynamic changes in the expression of the analysed markers in OECs and activation of the apoptotic pathway were observed. The data suggest that radiofrequency electromagnetic fields might play different and important roles in the self-renewal of OEC stem cells, which are involved in nervous system repair.

Keywords: Non-thermal effect, Amplitude modulation, Non-ionizing radiation, Olfactory glial cells, Cytoskeletal protein expression, Neural plasticity

INTRODUCTION

Until a few decades ago, it was believed that the damage produced in biological matter by electromagnetic radiation was related only to the tissue overheating, the so-called thermal effect, or to the capacity of the radiation to ionize the matter. The latter case requires an electromagnetic field (EMF) frequency about one million times higher than the radiofrequency electromagnetic field (RF-EMF). So, it was accepted that the only effect attributable to RF-EMFs was due to local tissue overheating, which requires a considerable intensity of the fields. Recently, it has been questioned (Havas, 2017) whether the ionizing radiation model is appropriate to explain the effect of a low-intensity RF-EMF. In fact, evidence of free-radical damage has been reported among humans, animals, plants and microorganisms produced by non-ionizing RF-EMFs (Cucurachi et al., 2013). The RF-EMF mechanisms of action are not understood, nor is their connection with the characteristics of the impinging electromagnetic field, e.g. specific absorption rate, exposure time (chronic or acute), carrier frequency, type of modulation. In this context, a useful investigation strategy could be to expose cells to EMFs under similar experimental conditions, but changing one of their characteristics (e.g. modulation).

Over the last few years, olfactory ensheathing cells (OECs), a particular glial cell type (Campisi et al., 2012), have attracted considerable interest as a promising tool for cellular therapy in spinal cord injury (Mackay-Sim and St John, 2011; Raisman et al., 2011; Zhang et al., 2017) and axonal growth (Chehrehasa et al., 2010), as they are able to stimulate axonal regeneration and functional restoration in lesions of the nervous system (Barnett and Riddell, 2004; Fairless and Barnett, 2005). They also share phenotypic properties with both Schwann cells and astrocytes (Ramón-Cueto and Avila, 1998). In addition, they are a source of growth factors, such as nerve growth factor, fibroblast growth factor, brain derived neurotrophic factor, glial derived neurotropic factor (Mackay-Sim and St John, 2011; Woodhall et al., 2001), and adhesion and extracellular matrix molecules (Franssen et al., 2007). They are also considered to be stem cells, as they express nestin (Oprych et al., 2017; Ramón-Cueto and Avila, 1998) and exhibit some particular characteristics, such as plasticity (Pellitteri et al., 2010; Yang et al., 2015). All these characteristics render them useful as an approach in cellular therapy (Pellitteri et al., 2016). It has also been reported that an early marker of Alzheimer and Parkinson diseases is the loss of olfactory performance (Attems et al., 2014).

Herein, for the first time in our knowledge, we assessed the effect of low-intensity EMFs on OEC cytoskeletal proteins. Cells were exposed to EMFs at continuous or amplitude-modulated (at 50 Hz) 900 MHz frequency, in the far-field condition and for different exposure times (10, 15, 20 min) under similar experimental specifications (intensity, carrier frequency). Therefore, we studied the cellular response in the simplest case: sinusoidal amplitude modulation. In particular, we evaluated, through immunocytochemical procedures, the expression of glial fibrillary acidic protein (GFAP), vimentin and nestin, which are proteins implicated in the formation of intermediate filaments, responsible for the cellular response in nervous system injury (Pellitteri et al., 2017; Pekny and Nilsson, 2005). The effect on cell viability was also tested. In addition, the positivity of cells to S-100 protein, a specific marker for OECs, and the involvement of activation of the apoptotic pathway were evaluated.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle's Medium (DMEM), heat-inactivated fetal bovine serum (FBS), normal goat serum (NGS), penicillin–streptomycin solution, 200 mmol l^{-1} L-glutamine, collagenase, and 0.05% trypsin–0.02% EDTA solution were obtained from Invitrogen (ThermoScientific, Milan, Italy). Cytosine arabinoside, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT), Lab-Tek™ Chamber Slides II and mouse monoclonal antibody against S-100 β subunit protein (S 2857) were obtained from Sigma-Aldrich (Milan, Italy). Monoclonal mouse anti-vimentin clone V9 (2012-03) and polyclonal rabbit anti GFAP (2015-02) were from DAKO. Rabbit anti-nestin polyclonal antibody was from Immunological Sciences (AB-10115). Mouse monoclonal antibody against caspase-3 was from Becton-Dickinson (611048). Cy3 goat anti-mouse IgG (115-165-145) and Cy3 goat anti-rabbit IgG (111-165-003) were from Immunological Research.

Animals

Experiments were performed on 2 day old mouse pups (P_2, provided by Envigo RMS s.r.l. Italy, stock: C57BL6J). Animals were kept in a controlled environment (23±1°C, 50±5% humidity) with a 12 h:12 h light:dark cycle with food and water available *ad libitum*. All the experimental procedures were carried out according to the Italian Guidelines for Animal Care (D.L. 116/92 and 26/2014), which are in compliance with the European Communities Council Directives (2010/63/EU) and were approved by the Ethical Committee at the University of Catania (Catania, Italy). Efforts were made to minimize the number of animals used and their suffering.

Primary OEC cultures

Primary OECs were isolated from mouse P_2 olfactory bulbs as previous described (Pellitteri et al., 2007). Briefly, the pups were decapitated and the bulbs were removed. Then, they were digested by collagenase and trypsin. Trypsinization was stopped by adding DMEM supplemented with 10% heat-inactivated FBS, 2 mmol l^{-1} glutamine, streptomycin (50 µg ml^{-1}) and penicillin (50 U ml^{-1}). Cells were resuspended and plated in flasks with fresh complete medium. The antimitotic agent cytosine arabinoside (10^{-5} mol l^{-1}) was added 24 h after. To further purify them, OEC cultures were processed to an additional step by transferring cells from this flask to a new one (Chuah and Au, 1993; Pellitteri et al., 2016). In the last passage, OECs were plated in 25 cm^2 flasks and cultured in DMEM/FBS supplemented with bovine pituitary extract. Cells were then incubated at 37°C in humidified air containing 5% CO_2 with a fresh complete medium and were fed twice a week.

Exposure of OECs to EMFs

Purified OECs were re-plated in complete culture medium on both 96 multiwell flat-bottomed 200 µl microplates and 14 mm diameter glass coverslips at a final density of 1×10^4 cells per well and 1×10^4 cells per coverslip, respectively, and incubated for 24 h at 37°C in humidified air containing 5% CO_2. For the experiments, cells were divided into four groups: cells maintained in the incubator at 37°C in an environment of humidified air and CO_2, in order to verify normal cellular status (control); cells floated in a thermal water bath at 37±0.1°C for 10, 15 and 20 min (sham); cells floated in a thermal water bath and exposed to EMFs for 10, 15 and 20 min at a continuous frequency of 900 MHz (CW 900 MHz); cells floated in a thermal water bath and exposed for 10, 15 and 20 min to an amplitude-modulated electromagnetic field with a carrier wave at 900 MHz and a sinusoidal amplitude modulation signal at 50 Hz (AM 900 MHz). The CW 900 MHz EMF was generated by an Agilent-8648D signal generator, amplified using a linear amplifier (provided by Temix Engineering s.r.l.) and emitted by a double horn antenna (ETS-Emco-3115). The AM 900 MHz EMF was generated by Agilent-8648D signal generator driven by a sinusoidal waveform at 50 Hz (modulation index=0.27) generated by an external source (Philips-PM5127); the amplitude-modulated EMF was amplified and emitted by the double horn antenna. The irradiation was performed from the top and the distance between the antenna aperture and the sample was 67 cm and therefore in the far-field condition. In both irradiation types, the electric field amplitude was about 7 V m^{-1} at the sample. Electric field amplitude was measured using a field meter (PMM 8053, Narda Safety Test Solutions), the EMF spectrum emitted by the antenna was monitored using a Biconical antenna (Austrian Research Centers, Seibersdorf, Austria). No significant variation in the uniformity of the electrical field in a region of 9 cm×13 cm around the centre of the sample holder was observed. After 20 min exposure time, an increase of temperature of less than 0.35°C was measured, so no change in the cell cultures induced by a thermal effect was envisaged. Before the experiment began, the occurrence of further exogenous electromagnetic fields was also checked in the range 5 Hz to 3 GHz. After exposure, cells were processed for MTT assay and for immunocytochemical procedures (Campisi et al., 2012).

MTT assay

To monitor cell viability, OECs were plated on 96 multiwell flat-bottomed 200 µl microplates as previously reported (Campisi et al., 2012; Pellitteri et al., 2017). The optical density of each well sample was measured with a microplate spectrophotometer reader (Titertek Multiskan, Flow Laboratories, Helsinki, Finland) at λ=570 nm. All experiments were performed in quadruplicate and the relative cell viability was expressed as a percentage with respect to control cells. Values are reported as means±s.d.

Immunocytochemical procedures

The expression of vimentin, GFAP, S-100 protein, nestin and caspase-3 cleavage protein was assessed through immunocytochemical procedures (Pellitteri et al., 2017). Cells, seeded on coverslips, were fixed by exposure to 4% paraformaldehyde with 0.1% Triton-X PBS for 30 min. OECs were successively incubated overnight in the following primary antibodies: mouse monoclonal antibody against vimentin (diluted 1:50), polyclonal rabbit antibody against GFAP (diluted 1:1000), mouse anti S-100 monoclonal antibody (diluted 1:100), rabbit polyclonal antibody against nestin (diluted 1:100) and mouse monoclonal antibody against caspase-3. Cells were successively incubated for 1 h at room temperature in the dark, in secondary antibody: Cy3 goat anti-rabbit IgG (diluted 1:200) to visualize nestin and GFAP, and Cy3 goat anti-mouse IgG (diluted 1:200) to visualize all other primary antibodies. Coverslips were then washed three times in PBS and mounted with PBS/glycerol. The immunostained coverslips were analysed using a Zeiss fluorescence microscope (Zeiss, Germany) and images were captured with an Axiovision Imaging System. The immunolabelled cells were counted in 10 different microscopic fields (20× magnification) and the positivity for each marker was expressed as a percentage and successively compared with each respective control. No non-specific staining of OECs was observed in control incubations in which the primary antibodies were omitted.

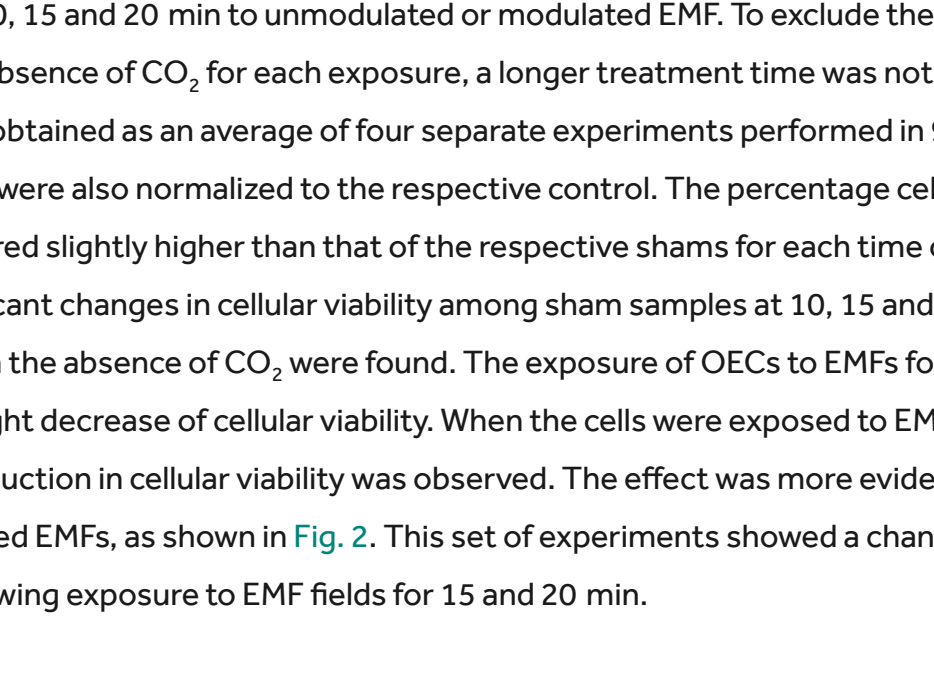
Statistical analysis

Data were statistically analysed using one-way analysis of variance (ANOVA) followed by a *post hoc* Holm–Šidák test to estimate significant differences among groups. Data are reported as means±s.d. of four separate experiments in duplicate, and differences between groups were considered to be significant at $P<0.05$, $P<0.01$ and $P<0.001$ as indicated in the figures.

RESULTS

The present *in vitro* study investigated the effect of short-term exposure (10, 15 and 20 min) of primary mouse OECs to EMFs on cell viability and the expression of vimentin, GFAP, S-100 protein and nestin through immunocytochemistry on single cells. Furthermore, the involvement of apoptotic pathway activation was also evaluated by testing caspase-3 cleavage. OECs were divided into four groups: control, sham, exposed to continuous 900 MHz (CW 900 MHz) and exposed to amplitude-modulated 900 MHz (AM 900 MHz). During each exposure, a group of OEC cell cultures was maintained in an incubator at 37°C as a control, in order to evaluate possible changes in the morphological cellular status versus those of cells (sham and exposed to EMFs) placed in thermal water bath at 37°C in the absence of CO_2. The changes in cellular morphology of OECs during exposure of the cells to EMFs were evaluated through phase-contrast inverted microscopy (Fig. 1). No significant differences between control and sham treatments at 10 and 15 min were observed, while a slight variance was observed with the 20 min exposure. No significant morphological change in OECs exposed for 10 min to CW 900 MHz or AM 900 MHz was found when the cells were compared with the respective controls and shams. A progressive hypertrophy between 15 and 20 min exposure of OECs to CW 900 MHz was observed, when compared with the respective controls and shams. The effect was more evident when the cells were exposed for 20 min. In OECs exposed to AM 900 MHz for 15 and 20 min, progressive cell wrinkling and a reduction of their number were observed, when compared with the control, sham and CW 900 MHz samples.

Fig. 1.



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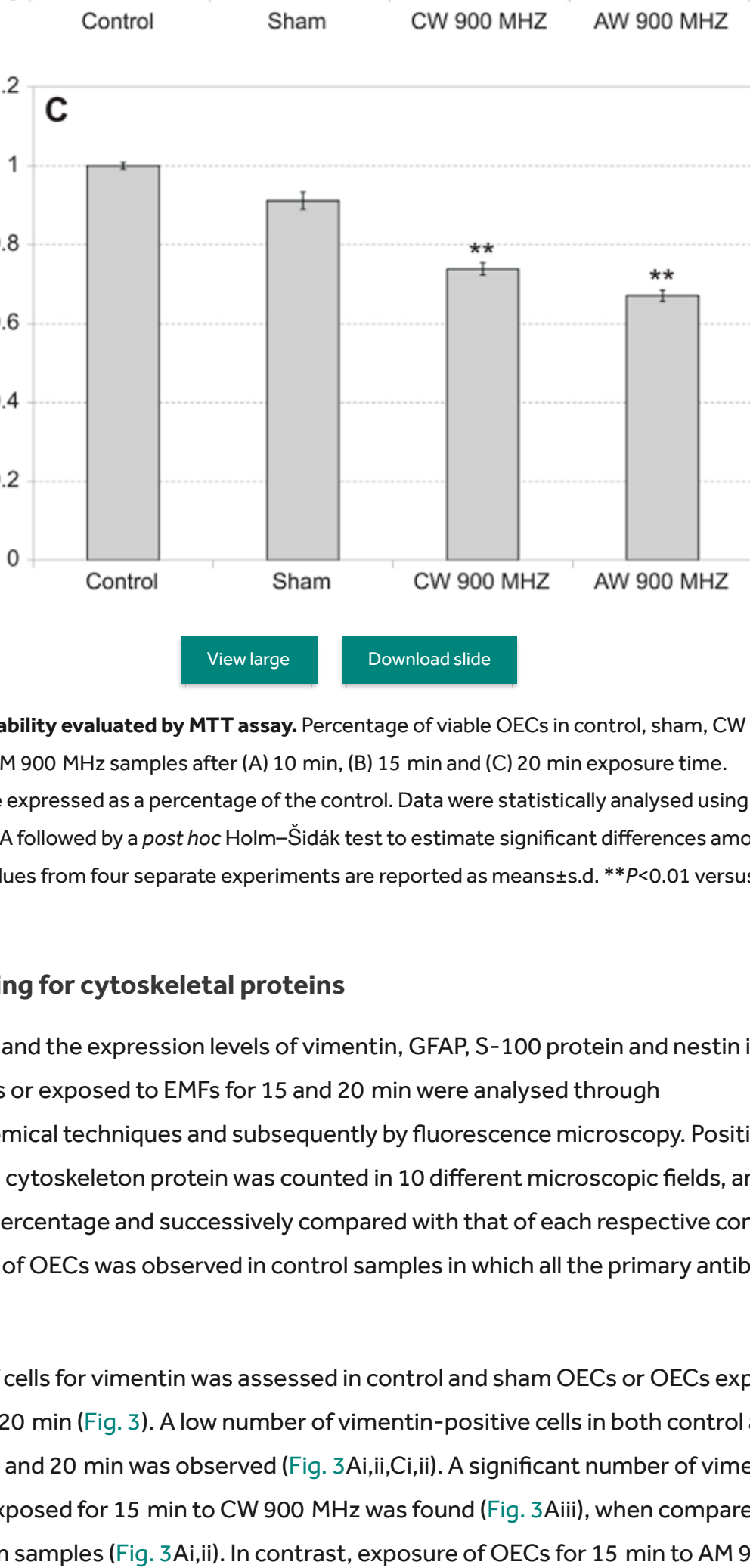
Qualitative analysis by phase-contrast inverted microscopy of representative fields of olfactory ensheathing cells (OECs) from the four treatments. (A) Control, and (B) sham, (C) CW 900 MHz and (D) AM 900 MHz samples after an exposure time of 10, 15 and 20 min. Magnification: 10×. Scale bars: 50 µm.

Cellular viability

To monitor cellular viability, the MTT test was used. Fig. 2 shows the percentage cell viability in OECs exposed for 10, 15 and 20 min to unmodulated or modulated EMF. To exclude the false positive due to the absence of CO_2 for each exposure, a longer treatment time was not used. The reported data were obtained as an average of four separate experiments performed in 96 multiwell plates and were also normalized to the respective control. The percentage cell viability in OEC controls appeared slightly higher than that of the respective shams for each time of exposure. No significant changes in cellular viability among sham samples at 10, 15 and 20 min of incubation at 37°C in the absence of CO_2 were found. The exposure of OECs to EMFs for 10 and 15 min induced a slight decrease of cellular viability. When the cells were exposed to EMFs for 20 min, a significant reduction in cellular viability was observed. The effect was more evident in OECs exposed to modulated EMFs, as shown in Fig. 2. This set of experiments showed a change in OEC cellular viability following exposure to EMF fields for 15 and 20 min.

Fig. 2.



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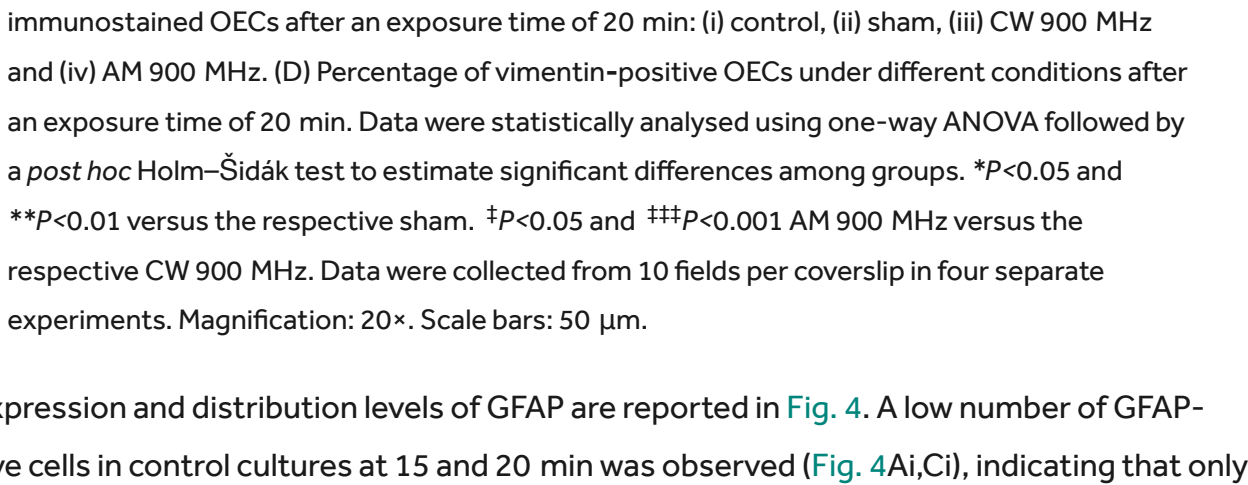
Cellular viability evaluated by MTT assay. Percentage of viable OECs in control, sham, CW 900 MHz and AM 900 MHz samples after (A) 10 min, (B) 15 min and (C) 20 min exposure time. Results are expressed as a percentage of the control. Data were statistically analysed using one-way ANOVA followed by a *post hoc* Holm–Šidák test to estimate significant differences among groups. Values from four separate experiments are reported as means±s.d. $^{**}P<0.01$ versus sham.

Immunolabelling for cytoskeletal proteins

The distribution and the expression levels of vimentin, GFAP, S-100 protein and nestin in OECs not exposed to EMFs or exposed to EMFs for 15 and 20 min were analysed through immunocytochemical techniques and subsequently by fluorescence microscopy. Positive cell number for each cytoskeleton protein was counted in 10 different microscopic fields, and expressed as a percentage and successively compared with that of each respective control. No specific staining of OECs was observed in control samples in which all the primary antibodies were omitted.

The positivity of cells for vimentin was assessed in control and sham OECs or OECs exposed to EMFs for 15 and 20 min (Fig. 3). A low number of vimentin-positive cells in both control and sham cultures after 15 and 20 min was observed (Fig. 3Ai,ii,Ci,ii). A significant number of vimentin-positive OECs exposed for 15 min to CW 900 MHz was found (Fig. 3Aiii), when compared with control and sham samples (Fig. 3Ai,ii). In contrast, exposure of OECs for 15 min to AM 900 MHz induced a slight decrease in the number of vimentin-positive cells (Fig. 3Aiv). As shown in Fig. 3Ciii, vimentin-positive immunostaining in OECs exposed to CW 900 MHz for 20 min appeared similar to that of OECs exposed for 15 min (Fig. 3Aiii). A very low number of OECs positive for vimentin after exposure to AM 900 MHz for 20 min was observed (Fig. 3Civ).

Fig. 3.



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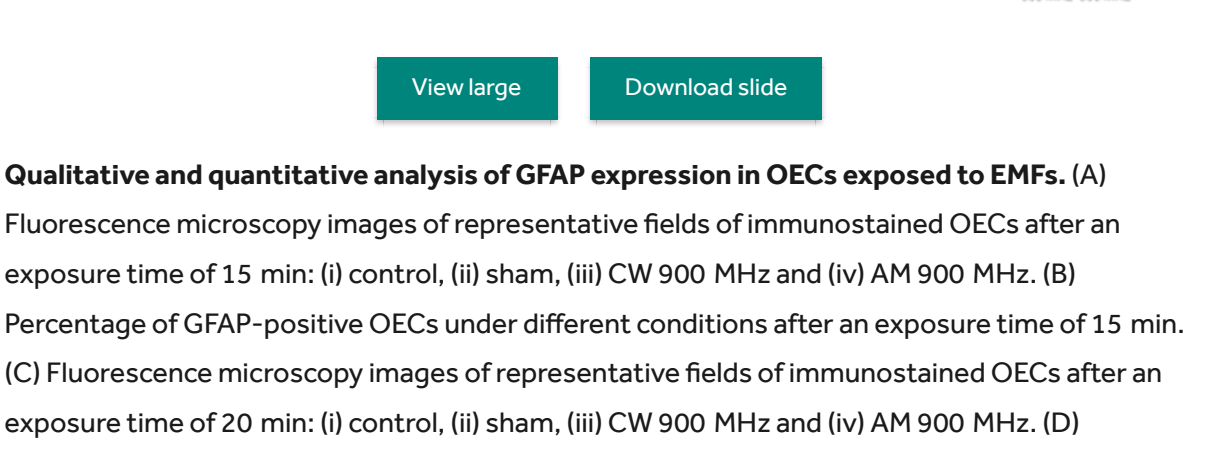
Qualitative and quantitative analysis of vimentin expression in OECs exposed to electromagnetic fields (EMFs). (A) Fluorescence microscopy images of representative fields of immunostained OECs after an exposure time of 15 min: (i) control, (ii) sham, (iii) CW 900 MHz and (iv) AM 900 MHz. (B) Percentage of vimentin-positive OECs under different conditions after an exposure time of 15 min. (C) Fluorescence microscopy images of representative fields of immunostained OECs after an exposure time of 20 min: (i) control, (ii) sham, (iii) CW 900 MHz and (iv) AM 900 MHz. (D) Percentage of vimentin-positive OECs under different conditions after an exposure time of 20 min. Data were statistically analysed using one-way ANOVA followed by a *post hoc* Holm–Šidák test to estimate significant differences among groups. $^{*}P<0.05$ and $^{**}P<0.01$ versus the respective sham. $^{‡}P<0.05$ and $^{‡‡‡}P<0.001$ AM 900 MHz versus the respective CW 900 MHz. Data were collected from 10 fields per coverslip in four separate experiments. Magnification: 20×. Scale bars: 50 µm.

The expression and distribution levels of GFAP are reported in Fig. 4. A low number of GFAP-positive cells in control cultures at 15 and 20 min was observed (Fig. 4Ai,Ci), indicating that only a few OECs were able to differentiate versus the astroglial cell type. A slight increase of GFAP-positive cells in sham cultures at 20 min was found in comparison with control (Fig. 4Ci,ii). An increase in GFAP levels was found when cells were exposed to CW 900 MHz at 15 and 20 min, when compared with control and sham samples (Fig. 4Aiii,Ciii). In contrast, exposure of OECs for 20 min to AM 900 MHz induced a decrease in the number of GFAP-positive cells compared not only with CW 900 MHz-exposed cells but also with control and sham samples (Fig. 4Civ). These results show that CW 900 MHz exposure induced gliosis, different from observations in cells exposed to AM 900 MHz and in both control and sham samples.

Fig. 4.



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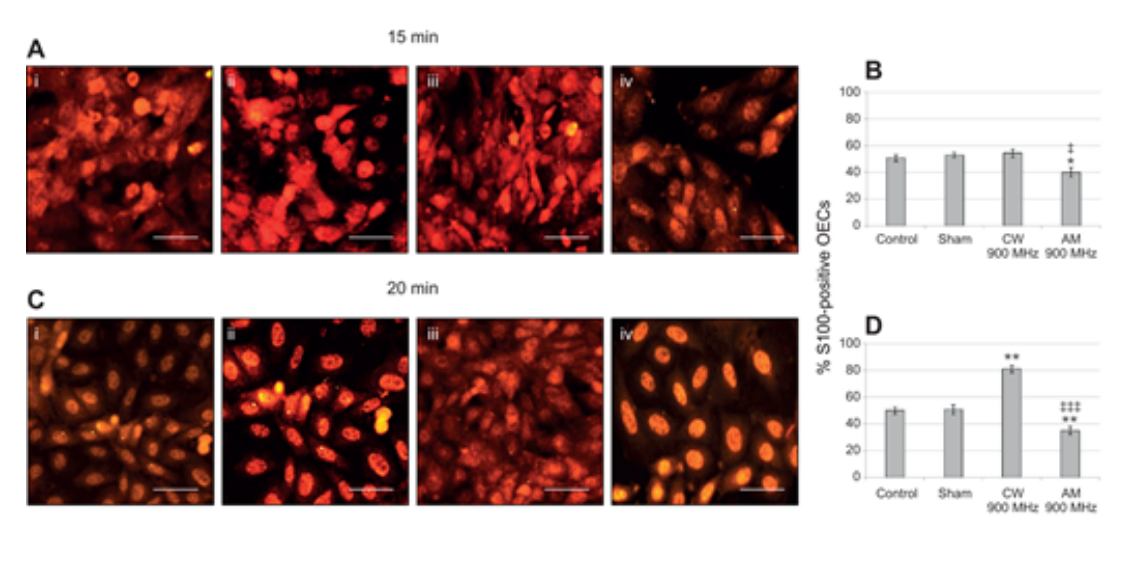
Qualitative and quantitative analysis of GFAP expression in OECs exposed to EMFs. (A) Fluorescence microscopy images of representative fields of immunostained OECs after an exposure time of 15 min: (i) control, (ii) sham, (iii) CW 900 MHz and (iv) AM 900 MHz. (B) Percentage of GFAP-positive OECs under different conditions after an exposure time of 15 min. (C) Fluorescence microscopy images of representative fields of immunostained OECs after an exposure time of 20 min: (i) control, (ii) sham, (iii) CW 900 MHz and (iv) AM 900 MHz. (D) Percentage of GFAP-positive OECs under different conditions after an exposure time of 20 min. Data were statistically analysed using one-way ANOVA followed by a *post hoc* Holm–Šidák test to estimate significant differences among groups. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ versus the respective sham. $^{‡‡‡}P<0.001$ AM 900 MHz versus the respective CW 900 MHz. Data were collected from 10 fields per coverslip in four separate experiments. Magnification: 20×. Scale bars: 50 µm.

Fig. 5 shows the number of positive cells for S-100 protein as a marker of OECs. No significant changes in the levels of the protein among the control, sham and CW 900 MHz samples exposed for 15 min were observed (Fig. 5Ai,ii,iii). In contrast, exposure of OECs for 15 and 20 min to AM 900 MHz induced a decrease in the number of S-100-positive cells (Fig. 5Aiv,Civ), when compared with the controls (Fig. 5Ai,Ci), shams (Fig. 5Aii,Cii) and CW 900 MHz OECs (Fig. 5Aiii,Ciii) exposed for 15 and 20 min, respectively. The effect was more evident in AM 900 MHz OECs exposed for 20 min (Fig. 5Civ). These data highlight that AM 900 MHz leads to OEC death, as indicated by a reduction in the number of S-100-positive cells.

Fig. 5.



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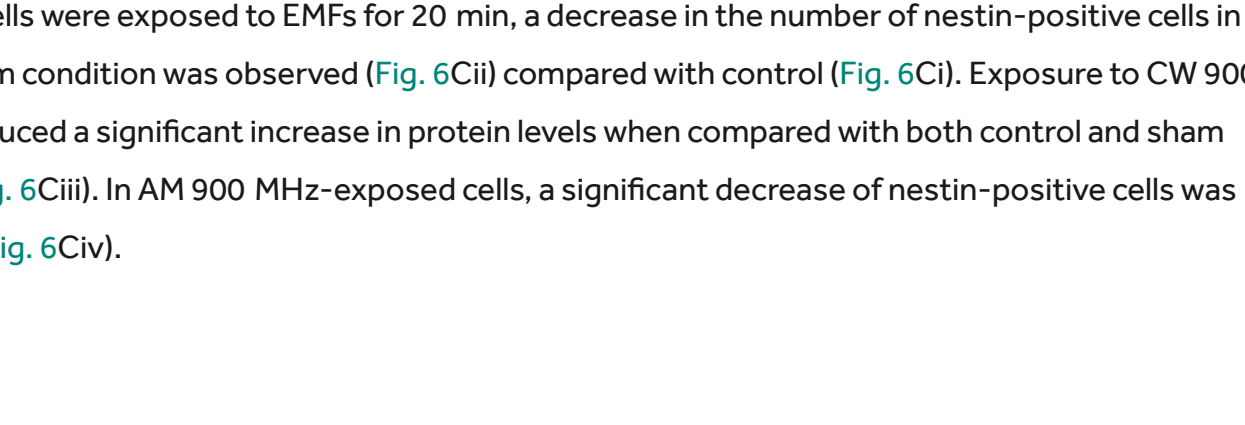
Qualitative and quantitative analysis of S-100 protein expression in OECs exposed to EMFs. (A) Fluorescence microscopy images of representative fields of immunostained OECs after an exposure time of 15 min: (i) control, (ii) sham, (iii) CW 900 MHz and (iv) AM 900 MHz. (B) Percentage of S-100-positive OECs under different conditions after an exposure time of 15 min. (C) Fluorescence microscopy images of representative fields of immunostained OECs after an exposure time of 20 min: (i) control, (ii) sham, (iii) CW 900 MHz and (iv) AM 900 MHz. (D) Percentage of S-100-positive OECs under different conditions after an exposure time of 20 min. Data were statistically analysed using one-way ANOVA followed by a *post hoc* Holm–Šidák test to estimate significant differences among groups. $^{*}P<0.05$ and $^{**}P<0.01$ versus the respective sham. $^{‡}P<0.05$ and $^{‡‡‡}P<0.001$ AM 900 MHz versus the respective CW 900 MHz. Data were collected from 10 fields per coverslip in four separate experiments. Magnification: 20×. Scale bars: 50 µm.

To assess whether exposure of OECs to EMFs was able to activate neural precursor stem cells, the expression and distribution of nestin, a neural stem cell marker, were evaluated. The same levels of nestin were expressed in all conditions at 15 min (Fig. 6A,B), although with very slight differences. When cells were exposed to EMFs for 20 min, a decrease in the number of nestin-positive cells in the sham condition was observed (Fig. 6Cii) compared with control (Fig. 6Ci). Exposure to CW 900 MHz induced a significant increase in protein levels when compared with both control and sham cells (Fig. 6Ciii). In AM 900 MHz-exposed cells, a significant decrease of nestin-positive cells was found (Fig. 6Civ).

Fig. 6.



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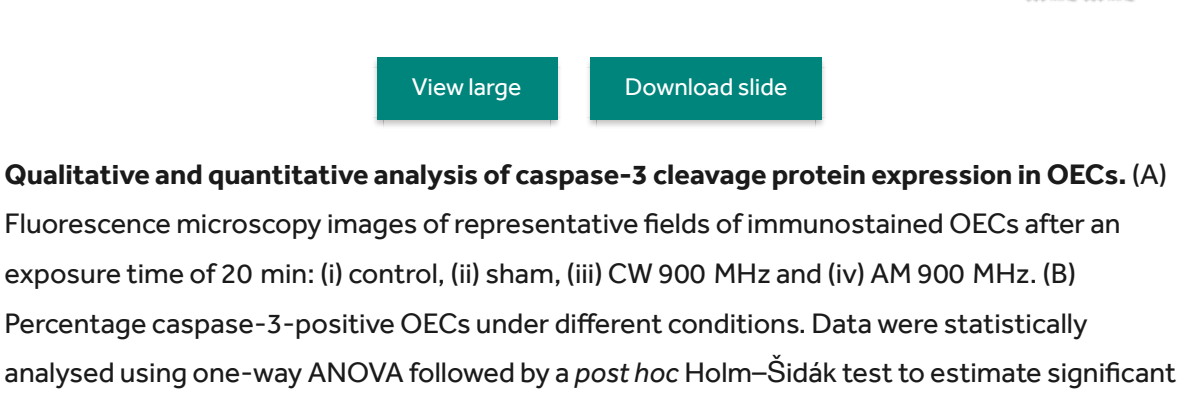
Qualitative and quantitative analysis of nestin protein expression in OECs exposed to EMFs. (A) Fluorescence microscopy images of representative fields of immunostained OECs after an exposure time of 15 min: (i) control, (ii) sham, (iii) CW 900 MHz and (iv) AM 900 MHz. (B) Percentage of nestin-positive OECs under different conditions after an exposure time of 15 min. (C) Fluorescence microscopy images of representative fields of immunostained OECs after an exposure time of 20 min: (i) control, (ii) sham, (iii) CW 900 MHz and (iv) AM 900 MHz. (D) Percentage of nestin-positive OECs under different conditions after an exposure time of 20 min. Data were statistically analysed using one-way ANOVA followed by a *post hoc* Holm–Šidák test to estimate significant differences among groups. $^{**}P<0.01$ and $^{***}P<0.001$ versus the respective sham. $^{‡‡‡}P<0.001$ AM 900 MHz versus the respective CW 900 MHz. $^{§§§}P<0.001$ sham versus the respective control. Data were collected from 10 fields per coverslip in four separate experiments. Magnification: 20×. Scale bars: 50 µm.

Immunolabelling for caspase-3 cleavage

Activation of the apoptotic pathway of caspase-3 cleavage was evaluated through immunocytochemical techniques both at 15 and at 20 min. No significant increases in caspase-3-positive cells were highlighted when OECs were exposed to EMFs in all experimental conditions for 15 min (data not shown). As shown in Fig. 7, in control, sham and CW 900 MHz samples exposed for 20 min, the number of OECs positive for caspase-3 was very low. A slight increase in caspase-3 expression in CW 900 MHz-exposed cells was found (Fig. 7Aiii), when compared with control and sham (Fig. 7Ai,ii). Differently from control and sham samples (Fig. 7Ai,ii), in CW 900 MHz-exposed OECs, caspase-3 was predominantly localized to the cytosol, with low levels in the nucleus (Fig. 7Aiii). In contrast, exposure of OECs for 20 min to AM 900 MHz induced a significant increase in the number of caspase-3-positive cells (Fig. 7Aiv) when compared with the control (Fig. 7Ai), sham (Fig. 7Aii) and CW 900 MHz (Fig. 7Aiii) samples. Caspase-3 in OECs exposed to AM 900 MHz for 20 min was localized both in the cytosol and in the nuclear compartment (Fig. 7D). These data demonstrate that AM 900 MHz exposure in OECs activates the apoptotic pathway, while unmodulated 900 MHz exposure induces a low activation of caspase-3 cleavage.

Fig. 7.



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Qualitative and quantitative analysis of caspase-3 cleavage protein expression in OECs. (A) Fluorescence microscopy images of representative fields of immunostained OECs after an exposure time of 20 min: (i) control, (ii) sham, (iii) CW 900 MHz and (iv) AM 900 MHz. (B) Percentage caspase-3-positive OECs under different conditions. Data were statistically analysed using one-way ANOVA followed by a *post hoc* Holm–Šidák test to estimate significant differences among groups. $^{*}P<0.05$ and $^{***}P<0.001$ versus the respective sham. $^{‡‡‡}P<0.001$ AM 900 MHz versus the respective CW 900 MHz. Data were collected from 10 fields per coverslip in four separate experiments. Magnification: 20×. Scale bars: 50 µm.

DISCUSSION

In the present *in vitro* study, we investigated the effect of acute exposure (10, 15 and 20 min) of primary mouse OECs to low-intensity RF-EMFs on cell viability, expression of some cytoskeletal proteins (vimentin, GFAP, S-100 protein and nestin) and the activation of the apoptotic pathway through immunocytochemical analysis. Cell cultures were exposed to continuous or amplitude-modulated (at 50 Hz) RF-EMFs in the far-field condition. In both experimental settings, the EMF was an electromagnetic wave at a frequency centred on 900 MHz. When modulated in amplitude, its envelope changed according to a sinusoidal law (50 Hz, modulation index 0.27). In previous research on differentiated primary rat neocortical astroglial cell cultures, we observed that exposure to amplitude-modulated 900 MHz EMF induced a reduction in cellular viability, an increase of intracellular reactive oxygen species (ROS) levels and DNA fragmentation (Campisi et al., 2010).

Despite the recent demonstration of the generation of non-thermal biological effects at the cellular level, there are still no adequate conclusive and persuasive results that correlate effects on health of exposure to RF-EMFs (Kaplan et al., 2016; Say et al., 2016). Electromagnetic radiation could induce structural damage affecting mitochondrial functionality, impairment of Ca^{2+} homeostasis and apoptosis (Hao et al., 2015; Xu et al., 2010).

Some reports show the effects of RF waves on growth, morphology, proliferation rate, DNA damage in mesenchymal stem cells, cancer cells and neurite outgrowth of embryonic neural stem cells in different experimental conditions (Barthélémy et al., 2016; Chen et al., 2014; Nikolova et al., 2005). However, there are few data on the effect of RF-EMFs on the self-renewal of neural progenitor cells (Shahbazi-Gahrouei et al., 2018), which play an important role in nervous system repair.

There is evidence on the impact of EMFs on the nervous system, depending on the cell type, including adult stem cells, and on heterogeneous characteristics of EMFs (e.g. intensity, exposure time, frequency, modulation) (Eghlidospour et al., 2017; Kim et al., 2013, 2019; Urnukhsaikhan et al., 2016).

EMF exposure might initiate a cascade of events by influencing the biology of living organisms, involving differential gene expression, differentiation and/or dedifferentiation, cellular proliferation, ROS production, apoptotic pathway activation and cell death (Arendash et al., 2010; Campisi et al., 2010; Şekeroğlu et al., 2012). It has been demonstrated that cell exposure to EMFs could be responsible for some tissue growth factor activation (Meyer et al., 2017; Sun et al., 2012) also involved in carcinogenesis (Kocaman et al., 2018) and in excitotoxicity (Campisi et al., 2010). Thus, to better understand the effect of EMFs on cellular plasticity, in this study we used OECs, a particular type of glial cell with stem cell characteristics (Pellitteri et al., 2016). Our choice was justified as OECs exhibit both plasticity (Campisi et al., 2010; Pellitteri et al., 2017; Yang et al., 2015) and the ability to secrete several growth factors (Pellitteri et al., 2010), which are responsible for promoting axonal regeneration and functional restoration in the injured central nervous system (Barnett and Riddell, 2004; Franssen et al., 2007; Ramón-Cueto et al., 2000). In addition, OECs are located in the olfactory system, which is the first to show a deficit in neurodegenerative diseases. To evaluate the modification of cytoskeletal marker expression induced by EMF exposure of OECs for 15 and 20 min, immunocytochemical techniques were used. A significant increase of GFAP expression – a marker of maturation, growth and differentiation of glia and a hallmark of reactive astrogliosis – was observed (Bouji et al., 2012; Eng, 1985; Schiffer et al., 1986).

It is well known that astrogliosis, which is usually correlated to brain development, is also achieved during glial scar formation as a result of traumatic phenomena, particularly during cortical wounding, brain injury, focal or global ischaemia and excitotoxicity (Campisi et al., 2003, 2012). The consequent cell morphology modifications, as progressive hypertrophy, reflect metabolic activation, as shown by increased organelle number or size, and the release of chemical factors

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(cytokines) in the case of mild to moderate astrogliosis (Schmidt-Kastner and Szymas, 1990; Sofroniew, 2009). The increase of GFAP was accompanied by an elevation of vimentin. This effect might be related to injury due to EMF exposure of OECs, which was particularly evident when the cells were exposed to CW 900 MHz for 20 min. Experimental evidence suggests that the increase in GFAP during reactive astrogliosis is mediated by an up-regulation of GFAP mRNA levels, as well as the vimentin changes (Calvo et al., 1991). Mechanisms of this response involve rearrangement of the intermediate filament network, including breakdown of F-actin stress fibres, and polymerization related to up-regulation of GFAP, vimentin and nestin.

In our experimental conditions, a significant number of nestin-positive cells in 900 MHz-exposed samples was found. The effect was more evident in CW 900 MHz cells exposed for 20 min. AM 900 MHz exposure for 20 min caused a significant decrease in nestin expression. Nestin is a marker of neural stem cells or progenitor cells that is also expressed by OECs (Doncel-Pérez et al., 2009), and it is transiently co-expressed in proliferating cells with both GFAP and vimentin during development and injury (Chou et al., 2003; Vanella et al., 2015). However, some authors have reported that after peripheral nerve injury in rodents, the number of nestin-expressing endoneural fibroblast-like cells significantly increased, leading to speculation that these cells may have progenitor-like properties (Richard et al., 2014). The effect observed between sham and control in nestin expression, which represents the earliest marker of neural stem cells, might be due to the stem cell characteristics of OECs.

As regards S-100 protein expression, a marker of OECs (Pellitteri et al., 2010), a significant increase was found when cells were exposed to 900 MHz at 20 min. Surprisingly, when OECs were exposed to AM 900 MHz, especially for 20 min, a decrease in the number of S-100 positive cells was observed. This last result highlights that AM 900 MHz might lead to cellular death as exposure reduced the number of S-100-positive OECs.

To verify the effects of EMF exposure on the activation of the apoptotic pathway, caspase-3 cleavage was assessed through immunocytochemical procedures. Our data showed a significant increase in caspase-3 cleavage protein expression in AM 900 MHz cells exposed for 20 min, indicating that in such cells the activation of the apoptotic pathway was induced. A small increase in caspase-3 cleavage in CW 900 MHz cells was observed. This last effect might be correlated with the stem cell characteristics of OECs, which are able to rapidly respond to EMF injury by proliferating, as highlighted by increases in nestin and vimentin expression. The observed effect might be related to the increase of intracellular Ca^{2+} levels in OECs, which mediate the organization of cytoskeletal proteins, modify the folding of some calcium-dependent proteins and activating the apoptotic pathway.

Conclusion

In conclusion, we firstly demonstrated that exposure of OECs, a type of stem cell, to EMFs for a brief time (20 min) induces a change in the organization of some cytoskeletal proteins (GFAP, vimentin, S-100, nestin), depending on the presence or absence of EMF amplitude modulation. In particular, we showed that cell exposure to CW 900 MHz for 20 min enhanced nestin expression levels, a neural stem cell marker expressed by OECs, stimulating self-renewal of the cells. Thus, CW 900 MHz exposure of OECs induced dynamic interactions among the expressed cytoskeleton proteins and the extrinsic signals induced from the microenvironment modification due to the exposure. This stimulates the intrinsic ability of OECs to repair the damage. In contrast, AM 900 MHz OEC exposure led to a significant reduction of cellular viability, as a result of activation of the apoptotic pathway, accompanied by a significant decrease in the tested cytoskeletal proteins. In addition, we suggest that CW 900 EMF might play an important role in the self-renewal of OECs, which is involved in the neural plasticity. Further investigations are now in progress in order to verify the hypothesis that CW 900 MHz and AM 900 MHz could induce different molecular mechanisms, which are able to promote repair and/or damage of OECs.

Footnotes

Author contributions

Conceptualization: R.G., R.P., F.M., G.R., A.S., A.T., A.C.; Methodology: R.G., R.P., A.C.; Validation: R.G., R.P., A.C.; Formal analysis: R.G., R.P., A.C.; Investigation: R.G., R.P., G.S., A.T., A.C.; Resources: R.G., R.P., S.A.C., F.M., G.R., A.S., A.C.; Data curation: R.G., R.P., A.C.; Writing - original draft: R.G., R.P., A.C.; Writing - review & editing: R.G., R.P., S.A.C., F.M., G.R., A.S., G.S., A.T., A.C.; Visualization: R.G., R.P., A.C.; Supervision: R.G., A.C.; Project administration: R.G., A.C.; Funding acquisition: R.G., F.M., G.R., A.S., A.T., A.C.

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Competing interests

The authors declare no competing or financial interests.

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